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The modality-specific contribution of peptidergic and non-peptidergic nociceptors is manifest at the level of dorsal horn nociresponsive neurons

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Key points

- Ablation of TRPV1+/peptidergic or of MrgprD+/non-peptidergic nociceptors produces modality-specific deficits in the behavioural responses to heat and mechanical stimuli, respectively. Noxious heat-induced dorsal horn Fos expression is also eliminated, despite the heat responsiveness of the non-peptidergic nociceptors.
- To assess whether this modality-specific contribution is manifest at the level of individual spinal neurons, we made extracellular recordings from mouse dorsal horn after selective ablation of the two nociceptor populations.
- Intrathecal capsaicin, which ablated the TRPV1+ nociceptors, abolished responsiveness of superficial and deep dorsal horn neurons to noxious heat, with no change in response to noxious mechanical stimulation.
- Ablation of MrgprD+ afferents did not alter the response to noxious heat but reduced the firing of dorsal horn neurons in response to noxious mechanical stimulation.
- These findings argue strongly that TRPV1+ and MrgprD+ nociceptors provide modality-specific contributions to the response properties of spinal cord neurons.

Abstract We previously demonstrated that genetic and/or pharmacological ablation of the TRPV1+/peptidergic or the MrgprD+/non-peptidergic subset of nociceptors produced selective, modality-specific deficits in the behavioural responses to heat and mechanical stimuli, respectively. To assess whether this modality-specific contribution is also manifest at the level of spinal cord neuron responsiveness, here we made extracellular recordings from lumbar dorsal horn neurons of the mouse in response to graded thermal and mechanical stimulation. We found that, following intrathecal injection of capsaicin to eliminate the central terminals of TRPV1+ nociceptors, neurons in the region of laminae I and V of the spinal cord lost responsiveness to noxious heat (whether generated by a contact heat probe or diode laser), with no change in their response to noxious mechanical stimulation. In contrast, ablation of MrgprD+ afferents did not alter the response to noxious heat, but reduced the firing of superficial dorsal horn nociceptive-specific neurons in response to graded mechanical stimulation and decreased the relative number of wide dynamic range neurons that were exclusively mechanosensitive. Neither ablation procedure reduced the number of dorsal horn neurons that responded to noxious cold. These findings support the conclusion that TRPV1+ nociceptors are necessary and probably sufficient for noxious heat activation of dorsal horn neurons and that, despite their polymodal properties,

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Abbreviations DTX, diphtheria toxin; HM, heat- and mechanosensitive; LTM, low-threshold mechanosensitive; M, selectively mechanosensitive; Mrgpr, Mrg family of G-protein-coupled receptors; NS, nociceptive specific; TRPAI, transient receptor potential ankyrin-repeat channel; TRPM8, transient receptor potential melastatin 8 channel; TRPV1, transient receptor potential vanilloid-1; WDR, wide dynamic range.

Introduction

There are two major subsets of unmyelinated primary afferent nociceptors: a transient receptor potential vanilloid-1-positive (TRPV1+), peptide-expressing population, and a non-peptidergic population that binds isolectin B4 and expresses the Mrg family of G-protein-coupled receptors (Mrgpr; Zylka *et al.* 2003, 2005; Basbaum *et al.* 2009). Of particular note is the MrgprD+ subpopulation, which comprises essentially all of the cutaneous, non-peptidergic afferents (Zylka *et al.* 2005). The peptidergic population targets dorsal horn projection neurons in lamina I and interneurons in superficial lamina II, while the non-peptidergic population primarily targets interneurons in lamina II (Basbaum *et al.* 2009). Our laboratory also provided evidence that these molecularly distinct nociceptor populations can be differentially activated by peripheral noxious stimuli (Braz & Basbaum, 2010) and engage different ascending circuits (Braz *et al.* 2005).

These anatomical differences are paralleled by modality-specific contributions of these nociceptor populations to the processing of pain messages. Thus, intrathecal injection of capsaicin, which ablates the central terminals of the TRPV1+ nociceptors, produced a near-complete loss of responsiveness to noxious heat (Cavanaugh *et al.* 2009), with no change in response to noxious mechanical or cold stimuli. Although Mishra *et al* (2011) reported that constitutive pharmacogenetic ablation of TRPV1+ neurons decreased responses to noxious cold, in addition to heat, the fact that they ablated TRPV1+ afferents in the embryo, when TRPV1 has a much broader distribution (Cavanaugh *et al.* 2011), is likely to account for this more extensive phenotype. In contrast, pharmacogenetic ablation of the MrgprD+ subset, by injection of diphtheria toxin (DTX) into adult mice in which the human diphtheria toxin receptor was knocked into the *MrgprD* locus, resulted in a selective reduction of mechanical sensitivity (Cavanaugh *et al.* 2009). Taking these results together, we concluded that the TRPV1+ and MrgprD+ populations of nociceptors contribute, respectively, to the nocifensive behaviours evoked by noxious heat and mechanical stimulation.

Not only was heat responsiveness largely eliminated after intrathecal capsaicin, but we also found a complete loss of noxious heat-induced Fos expression in the dorsal horn (Cavanaugh *et al.* 2009). As the TRPV1-negative population of nociceptors responds to both heat and mechanical stimulation (i.e. these nociceptors are polymodal; Stucky & Lewin, 1999; Rau *et al.* 2009), the inability to induce Fos expression was unexpected. Apparently, the integrated activity generated by noxious heat in the TRPV1-negative population is not sufficient to drive activity in dorsal horn nociresponsive neurons.

As Fos expression provides an indirect measure of neuronal activity, here we made extracellular recordings of nociresponsive neurons in the dorsal horn of wild-type mice and in mice in which either the TRPV1+ or MrgprD+ subpopulation was ablated. We found that the TRPV1+ subset of nociceptors is indeed required for noxious heat activation of both superficial and deep dorsal horn neurons, but not for mechanical responsiveness. In contrast, ablation of the MrgprD+ population selectively reduced responsiveness to noxious mechanical stimulation. Thus, despite the polymodal properties of the peptidergic and non-peptidergic neurons, the influence of these populations on nociceptive processing of dorsal horn neurons appears to be modality specific.

Methods

Ablation of subpopulations of afferents

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California San Francisco. Capsaicin and DTX pretreatments were conducted as described previously (Cavanaugh *et al.* 2009; Shields *et al.* 2010). In brief, to ablate the central terminals of TRPV1+ afferents, wild-type C57Bl/6 mice (20–30 g; Charles River, Wilmington, MA, USA) were anaesthetized with 1.5% isoflurane and then received an intrathecal injection of capsaicin (10 μg in 5.0 μl) or vehicle (10% ethanol and 10% Tween 80 in saline) over the lumbar enlargement. Success of the injection was confirmed by the complete loss of nocifensive behaviour in the 55°C hotplate test

on the following day. Animals that continued to respond were not studied further. To ablate MrgprD+ afferents, we injected DTX (100 μg kg^{-1}) intraperitoneally into mice in which the human diphtheria toxin receptor was knocked into the *MrgprD* locus (MrgprD$^{DTR/+}$; Cavanaugh *et al.* 2009). Injections were made on 2 days, separated by 72 h. Electrophysiological studies were performed by a blinded experimenter, at least 1 week and in some cases up to 6 weeks after the ablation protocol.

Electrophysiology

We performed extracellular single-unit recording from neurons of the superficial dorsal horn of the spinal cord, as described previously (Eckert *et al.* 2006; Mazario & Basbaum, 2007). Briefly, mice were anaesthetized with an intraperitoneal injection of urethane (1.5 g kg^{-1}, 10% in saline; Sigma). The adequacy of anaesthesia was regularly verified by continual heart rate monitoring and by the absence of a withdrawal reflex to a noxious pinch of the digits. Anaesthetic was supplemented as needed (0.2 g kg^{-1}). Dexamethasone (0.2 mg (50 μl)$^{-1}$; American Regent Laboratories, Shirley, NY, USA) and atropine (0.3 mg (300 μl)$^{-1}$; Sigma) were injected subcutaneously to minimize spinal cord swelling and to reduce secretions, respectively. A laminectomy was performed at vertebral level T13–L1, corresponding to spinal segments L4–L5. The mouse was then transferred to a specialized head holder, and the vertebral segments on both sides of the laminectomy were clamped. The dura was incised, and a spinal pool was formed with 5% agar and then filled with 37°C mineral oil. Core temperature was maintained close to 37°C with a circulating water pad. Mice breathed spontaneously throughout the experiment.

We first placed a fire-polished silver electrode over the lumbar enlargement while electrically stimulating the hindpaw. This search protocol identified the largest cord dorsum potential, which defined the locus for subsequent single-unit recording. Once an optimal recording site was located, we advanced a fine-tipped tungsten microelectrode with impedance of 6–8 MΩ at 1 kHz (FHC, Bowdoin, ME, USA) into the spinal cord using a motorized microdrive (FHC). To prevent sensitization of neurons following repeated heat stimulation, we used a mechanical search stimulus to locate responsive neurons. As we did not activate dorsal horn neurons antidromically, we cannot conclude unequivocally that we were recording from laminae I and V projection neurons. However, given the impedance of the electrodes and the relatively large size of the projection neurons, especially compared with the interneurons of lamina II, this seems likely. Presumptive lamina I neurons, therefore, were identified by applying brief, moderate pressure with a blunt glass probe to different regions of the glabrous skin of the ipsilateral hindpaw. Once a mechanical receptive field was identified, we tested the unit qualitatively for its responsiveness to brush (10 s), pressure (10 s), pinch (1–2 s) and a 50°C drop of water gently applied to the receptive field. Next, we used a 12 mm^2 polished metal probe (Estimec; Cibertec, Madrid, Spain) to apply graded mechanical stimuli (130, 230, 330 and 430 mN). Target intensity was reached within 0.1 s. Our protocol for classifying neurons as nociceptive specific (NS), wide dynamic range (WDR) or low-threshold mechanosensitive (LTM) is described in the Results section. We also applied a heat stimulus with a Peltier contact thermal device (40, 45 and 49°C, 9 mm^2 probe, 2.0°C s^{-1}; MSDRL, West Point, OA, USA) for 10 s with at least an 80 s interval between stimuli. The thermode was maintained at 36°C between stimuli. Finally, using the same Peltier device we tested several units for their response to innocuous (20°C) and noxious cold stimulation (10°C).

Laser thermal stimulation

For all units that responded to stimulation with the contact thermode, we also used an infrared diode laser (LASS-10 M; Lasmed, Mountain View, CA, USA; output wavelength of 980 nm) to irradiate the receptive fields and selectively activate C or Aδ heat-sensitive nociceptors. In addition, we sampled a total of 23 heat-insensitive units (11 in lamina I and 12 in lamina V) in capsaicin-treated mice so as to test the extent to which laser stimulation recapitulates findings obtained with noxious heat. The use of this laser met the safety requirements of the University of California San Francisco and was approved by the Office of Environmental Health and Safety of the University.

Previous studies found that cutaneous C fibres can be activated selectively by low-energy power, with a slow ramp and a larger beam diameter. The Aδ afferents can be activated selectively with a higher energy beam, with a more rapid rise time and a smaller spot diameter (Tzabazis *et al.* 2005; Cuellar *et al.* 2010; Mitchell *et al.* 2010). In the present study, we first used an aiming diode laser (630 nm, 5 mW maximum) to generate a visible spot of the appropriate diameter (5.0 mm for the C fibre protocol and 1.0 mm for the Aδ fibre protocol) over the receptive field. (As the laser beam is presented at an angle of approximately 70 deg to the skin, the spot was slightly elliptical. Spot diameter was, therefore, calculated from the minimal diameter of the ellipse.) In these experiments, the laser probe collimator was positioned at a fixed distance of 58 or 18 mm from the surface of the receptive field for C and Aδ afferent stimulation, respectively. Next, the stimulating laser beam (980 nm) was delivered through the laser collimator to the same spot for 2 s to activate C fibres and for 80 ms to activate Aδ fibres. The initial stimulation

intensity for C fibres was set at 300 mA, and this was increased in 50 mA steps until the unit fired (threshold). The receptive field was then stimulated with two more steps, at 50 and 100 mA above the threshold. If threshold could not be defined, then a cut-off of 1000 mA was established. Stimulation intensity for Aδ fibre activation was initiated at the C fibre threshold intensity, and then increased in 100 mA steps until the unit fired. This was defined as the Aδ threshold. The unit was then further stimulated at 100 and 200 mA above the threshold. A cut-off intensity of 4000 mA was set for Aδ stimulation.

To calibrate the temperature of the skin in response to laser stimulation, we used an infrared camera (ThermoVision SC6000; FLIR Systems, Inc., CA, USA) that was placed above the receptive field. As skin temperature recovered to normal within 20 s after termination of the laser stimulus, we set a 1 min interval between two laser stimuli. Average skin surface temperature for threshold activation in response to the C fibre laser protocol was 42°C. For Aδ stimulation, threshold activation corresponded to a surface temperature of approximately 58°C. Neither the C fibre nor the Aδ fibre laser protocol produced visible injury of the skin, and the responses to successive laser stimuli, presented at 1 min intervals and at the same intensity, did not differ.

Histology

After completing recordings, we made an electrolytic lesion by injecting current (10 μA for 5 s) to localize the recordings sites. The animals were perfused intracardially with 0.1 M phosphate-buffered 10% formalin. To visualize the recording sites, we stained 30 μm lumbar spinal cord sections with Cresyl Violet. Figure 1*D* illustrates electrode recording sites in the superficial and deep dorsal horn (in the region of laminae I and V, respectively). Typically, the depth of superficial dorsal horn recordings was ~70 μm from the cord surface; for the deep dorsal horn/lamina V, recordings were ~460 μm from the surface.

Data analysis

Unit activity was amplified, notch filtered (CyberAmp380; Axon Instruments), digitized [Micro1401; Cambridge Electronic Design (CED), Cambridge, UK] and discriminated (Spike2; CED). We recorded both the total number of spikes and the peak firing rate at the different heat and mechanical stimulus intensities (in 1 s bins). We used one-way ANOVA to analyse coding properties of the neurons, i.e. response to graded thermal, mechanical or laser stimuli, and used two-way ANOVA to compare differences between treatment groups in response to the heat or mechanical stimuli. Student's unpaired *t* test was used to compare mean recording depths between groups (GraphPad Prism; GraphPad Software Inc., La Jolla, CA, USA). Fisher's exact test or Z-test was used to compare the relative number or proportion, respectively, of spinal cord neuron types between groups (In-Silico.net, 2011). Data are presented as means ± SEM. Values of $P < 0.05$ were considered significant.

Results

Classification of dorsal horn neurons

We recorded a total of 172 neurons in the superficial dorsal horn, at a mean depth of 68.9 ± 4.0 μm, and 84 deep dorsal horn neurons, at a mean depth of 463.8 ± 4.7 μm from the surface of the cord. Neurons were classified as NS, WDR or LTM according to their responsiveness to mechanical stimulation. Units that responded to higher intensity mechanical stimulation (230, 330 and 430 mN), but not to the lowest intensity stimulation (brush and 130 mN), were classified as NS (for example, see Fig. 1*A*). Units that responded to both the lowest and highest intensity mechanical stimulation were considered WDR (for example, see Fig. 1*B*). Note that the latter neurons also responded to the innocuous glass probe, which confirms their WDR classification. Finally, neurons that were activated by innocuous mechanical stimulation but failed to encode stimulus intensities into the noxious range were considered LTM. Given that we used a mechanical search stimulus, all neurons in this study were mechanically sensitive. Thus, based on the stimulus modalities that activated them, neurons were classified as either selectively mechanosensitive (M; for example, see Fig. 1*B*), or heat- and mechanosensitive (HM; Fig. 1*A*).

Ablation of TRPV1+ afferents eliminates noxious heat-evoked activation of dorsal horn neurons, but does not alter mechanical coding

We recorded from 38 dorsal horn neurons in 26 animals that received an intrathecal injection of high-dose capsaicin, which ablated the central terminals of TRPV1+ neurons at lumbosacral levels (Cavanaugh *et al.* 2009). We were particularly interested in the extent to which TRPV1-negative afferents are able to evoke neuronal firing in response to noxious heat stimulation. We also recorded from 61 neurons in 30 vehicle-treated mice.

In agreement with previous studies in rats and cats (Light, 1992; Urch *et al.* 2003), we found that NS neurons predominated in the superficial dorsal horn of vehicle-treated mice. Thus, 62.3% (38 of 61) of superficial dorsal horn neurons from vehicle-treated animals were classified as NS, compared with only 32.8% (20 of 61) that had a WDR phenotype (Fig. 2*A*). These relative proportions were unchanged following ablation of TRPV1+ afferents, because 57.9% (22 of 38) of recorded

neurons were NS and 31.6% (12 of 38) were WDR in capsaicin-treated mice (Fig. 2*A*). In addition, a very small number of cells in each group of mice were LTM neurons (3 of 61 and 4 of 38 for vehicle- and capsaicin-treated mice, respectively).

In vehicle-treated mice, we found that the vast majority of superficial dorsal horn neurons (68.9%; 42 of 61) responded to a 50°C noxious heat stimulus (Fig. 2*A*). In sharp contrast, superficial dorsal horn neurons were virtually non-responsive to noxious heat stimulation following capsaicin treatment. Thus, only 2 of 38 cells responded to noxious heat in capsaicin-treated mice (1 NS and 1 WDR; see Fig. 2*B*; $P < 0.0001$ compared with vehicle-treated mice by Fisher's exact test). Moreover, the unusual firing properties of these two responsive neurons suggest that they resulted from an incomplete effect of the capsaicin treatment. For example, although the firing threshold (45°C) of both neurons was comparable to that of the vehicle-treated animals, both the peak firing rate and total number of spikes evoked by the 49°C stimulus were considerably lower, and lacked the intensity coding generally observed in vehicle-treated mice.

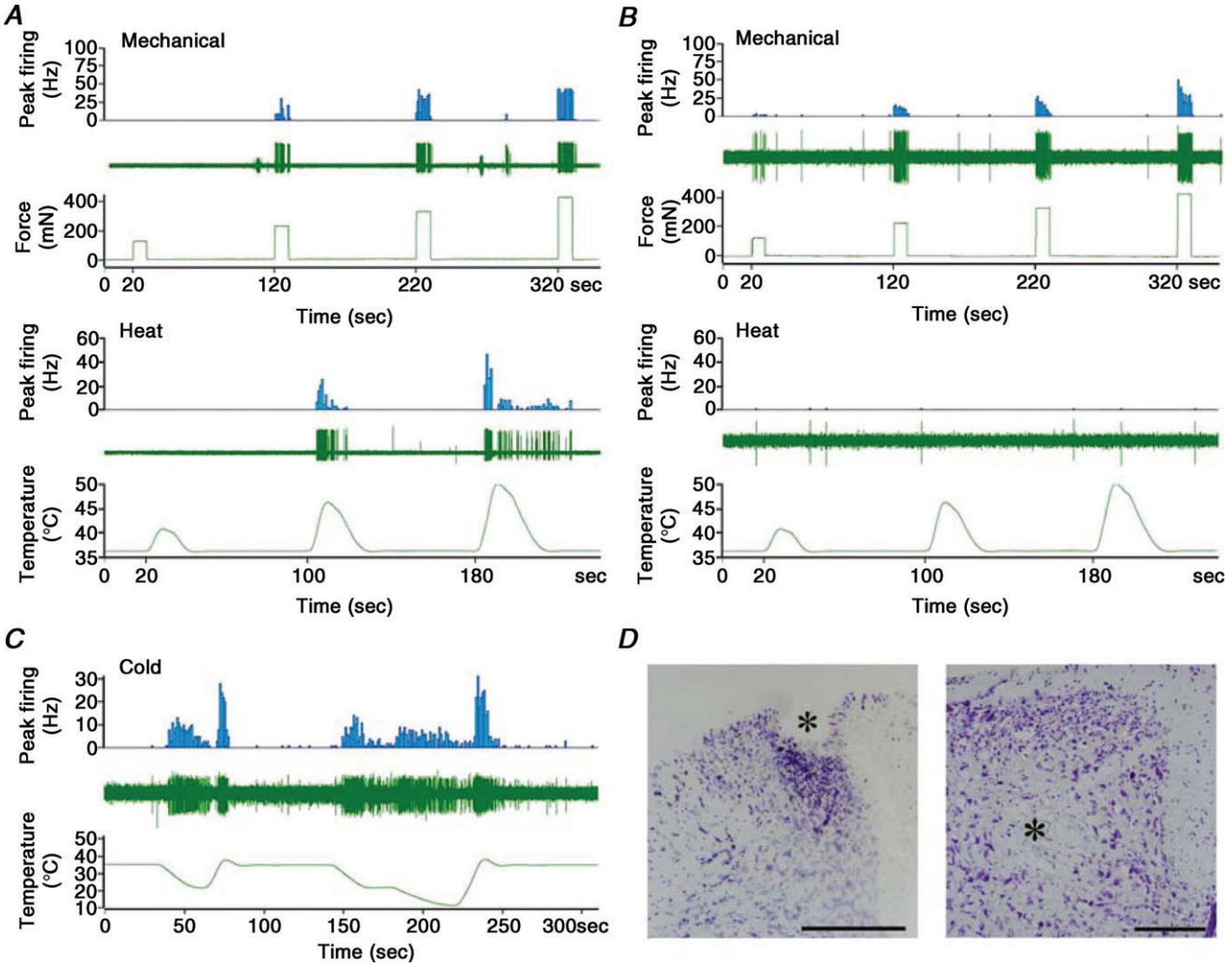
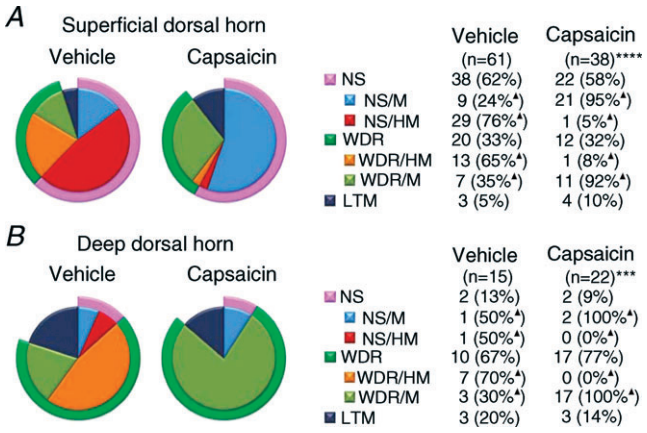


Figure 1. Nociresponsive neurons recorded from superficial and deep dorsal horn of the spinal cord
A, example of a superficial dorsal horn nociceptive specific (NS) neuron that could be activated by graded mechanical (upper three traces) and heat stimulation (lower three traces) of the hindpaw. Each record contains three traces: firing rate (top); raw data (middle); and stimulus intensities (bottom; 130, 230, 330 and 430 mN for mechanical and 40, 45 and 49°C for heat). The *x*-axis defines the temporal stimulus sequence (in seconds). Note that the neuron did not discharge in response to the innocuous 130 mN stimulus. *B*, example of a lamina I wide dynamic range (WDR) neuron that responded only to mechanical stimulation, including the 130 mN stimulus. *C*, example of a deep dorsal horn neuron that responded to innocuous (20°C) and noxious (10°C) cold stimuli. This particular neuron also discharged when the temperature of the receptive field returned to baseline (36°C). *D*, recording sites (∗) in the superficial (left panel) and in the deep dorsal horn (right panel). Scale bars represent 250 μm.



	Vehicle (n=61)	Capsaicin (n=38)****
NS	38 (62%)	22 (58%)
NS/M	9 (24%▲)	21 (95%▲)
NS/HM	29 (76%▲)	1 (5%▲)
WDR	20 (33%)	12 (32%)
WDR/HM	13 (65%▲)	1 (8%▲)
WDR/M	7 (35%▲)	11 (92%▲)
LTM	3 (5%)	4 (10%)

	Vehicle (n=15)	Capsaicin (n=22)***
NS	2 (13%)	2 (9%)
NS/M	1 (50%▲)	2 (100%▲)
NS/HM	1 (50%▲)	0 (0%▲)
WDR	10 (67%)	17 (77%)
WDR/HM	7 (70%▲)	0 (0%▲)
WDR/M	3 (30%▲)	17 (100%▲)
LTM	3 (20%)	3 (14%)

Figure 2. Categorization of mechanically responsive superficial and deep dorsal horn neurons in vehicle- and capsaicin-treated mice
A, these multilevel pie charts schematize the relative abundance of the different categories of lamina I neurons in vehicle- and capsaicin-treated mice. The percentage relative to the total number of neurons recorded is listed in the adjacent table. Note the near-complete loss of heat-responsive neurons following capsaicin treatment (****$P < 0.0001$, Fisher's exact test). *B*, these multilevel pie charts schematize the relative abundance of the different categories of deep dorsal horn neurons, with their percentage relative to the total number of neurons recorded listed in the adjacent table. As for superficial dorsal horn neurons, capsaicin treatment eliminated heat responses of lamina V neurons (***$P < 0.001$, Fisher's exact test). Percentages marked with a filled triangle refer to the relative number of NS/M or NS/HM in the NS group, or of WDR/M or WDR/HM in the WDR group. Abbreviations: HM, heat- and mechanosensitive; LTM, low-threshold mechanosensitive; M, selectively mechanosensitive; NS, nociceptive specific; and WDR, wide dynamic range.

In contrast, we found no change in the mechanical responsiveness of the superficial dorsal horn neurons recorded following capsaicin treatment. Thus, superficial dorsal horn neurons from vehicle- and capsaicin-treated mice were indistinguishable with respect to either the peak firing rate or the total number of spikes evoked by a range of noxious mechanical stimulus intensities (Fig. 3*A* and *B*).

Results from recordings in the deep dorsal horn (15 neurons in vehicle-treated mice and 22 in capsaicin-treated mice) paralleled those in the superficial dorsal horn. Thus, as expected from the studies in the mice with a *Trpv1* deletion (Eckert *et al.* 2006), we found that these neurons were unresponsive to noxious heat after intrathecal capsaicin (8 of 15 neurons were heat responsive in vehicle-treated mice, compared with 0 of 22 neurons in capsaicin-treated mice; $P < 0.001$, Fisher's exact test; Fig. 2*B*). As for neurons in the superficial dorsal horn, in the deep dorsal horn we found no change in the responsiveness to innocuous or noxious mechanical stimulation. Coding to mechanical stimulation was comparable, with respect to both peak firing rates and total number of spikes as a function of stimulus intensity (Fig. 3*C*). Taking these results together, we conclude that the ability of TRPV1-negative afferents to respond to noxious heat is not sufficient to activate dorsal horn WDR or NS neurons (in the superficial or deep dorsal horn) in the absence of TRPV1+ afferents. Furthermore, loss of the TRPV1+ population does not result in a change in the mechanical responsiveness of dorsal horn nociresponsive neurons. Thus, any putative

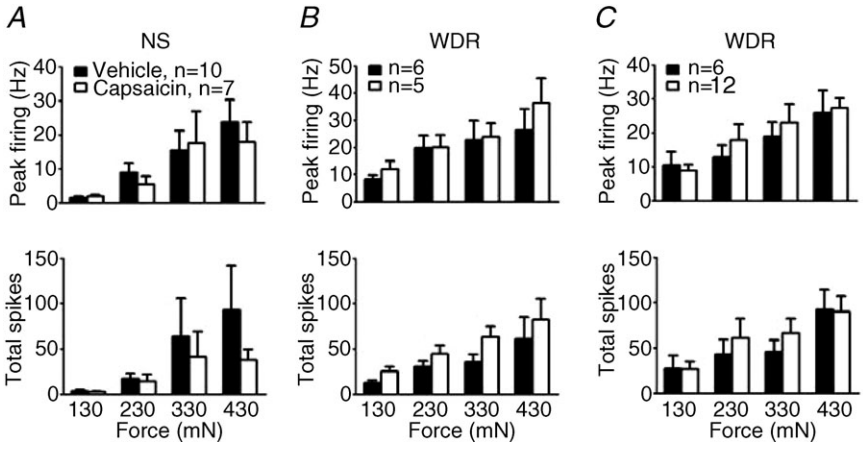


Figure 3. Ablation of transient receptor potential vanilloid-1-positive (TRPV1+) afferents does not alter the mechanical responsiveness of spinal cord nociresponsive neurons
Peak firing (top panels) and total spikes (bottom panels) elicited by mechanical stimulation at the indicated intensities, for superficial dorsal horn NS neurons (*A*), superficial dorsal horn WDR neurons (*B*) and deep dorsal horn WDR neurons (*C*) in vehicle-treated (filled columns) and capsaicin-treated mice (open columns). No significant differences were observed for the mechanical responsiveness between vehicle- and capsaicin-treated animals in any of these cell subtypes ($P > 0.05$, two-way ANOVA). For all neuronal types, both the peak firing rate and the total number of spikes significantly increased in response to increased mechanical stimulus intensity (one-way ANOVA). Data are presented as means + SEM.

polymodal contribution of this population is not manifest at the level of dorsal horn responsiveness.

Comparison of heat and laser responsiveness of lamina I neurons

The noxious heat-induced activity of dorsal horn nociresponsive neurons derives from inputs carried by TRPV1+ C and Aδ nociceptors. Although there have been attempts to activate Aδ and C nociceptors selectively with different heat stimulation protocols (Yeomans & Proudfit, 1996), the parameters for selective activation are more readily achieved using a diode laser than radiant or contact heat stimuli. For this reason, we also assessed the responsiveness of dorsal horn neurons using diode laser-induced activation of C and Aδ nociceptors (Tzabazis *et al.* 2005). The selectivity of the two laser protocols was confirmed by the generation of short and long response latencies for activation of spinal cord neurons in response to the Aδ fibre and C fibre laser protocols, respectively (Fig. 4*B* and *D*)

We recorded a total of 33 presumptive lamina I cells in vehicle-treated mice, including 25 heat-responsive and eight heat-insensitive cells. After characterizing the responsiveness to the contact heat probe, we stimulated the receptive field with a laser protocol programmed to activate only C fibres (see Methods). We found that all 25 noxious heat-responsive cells in vehicle-treated mice responded to the laser stimulus with an activation threshold of 459 ± 34 mA ($n = 25$); none of the eight heat-insensitive cells responded. We then examined the response of the neurons to two 50 mA step increases of the laser current above the firing threshold and found that the neurons coded for increased intensity by increasing their peak firing rate (2.89 ± 0.71, 5.39 ± 0.82 and 11.16 ± 2.23 Hz in response to the three steps of stimulation intensities; $P < 0.001$, one-way ANOVA; Fig. 4*A*). Figure 4 also shows that as the current intensity increased, the response latencies progressively decreased (1.99 ± 0.14, 1.64 ± 0.08 and 1.31 ± 0.12 s; $P < 0.01$, one-way ANOVA; Fig. 4*B*).

Following this, we tested these heat-responsive cells with the Aδ stimulation protocol. As some of the cells were lost during the recording time, we studied only 17 of the 25 heat-responsive neurons. We found that 13 of these 17 cells (all of which responded to the C fibre protocol) could be activated by the Aδ laser stimulation protocol, with a mean activation threshold of 2317 ± 251 mA. Step increases of 100 mA above the threshold further showed that the neurons coded for stimulation intensity with an increased peak firing rate (2.14 ± 1.01, 5.43 ± 2.50 and 9.15 ± 3.70 Hz for the three steps; $P < 0.05$, one-way ANOVA; Fig. 4*C*). As for the C fibre protocol, the response latencies decreased at higher Aδ laser stimulation intensities (106.7 ± 9.1, 82.7 ± 13.3 and 63.4 ± 12.8 ms; $P < 0.01$, one-way ANOVA; Fig. 4*D*).

Taken together, these results demonstrate that the heat input to heat-responsive neurons in the superficial dorsal horn derives predominantly from convergent C and Aδ primary afferent input. All neurons receive a C fibre input, and approximately 75% also respond to Aδ afferent stimulation. It is noteworthy that as our search strategy involved initial mechanical stimulation, these data represent the responsiveness of polymodal (including both wide dynamic range and nociceptive-specific) neurons. Whether cells that respond only to noxious heat stimulation receive a different complement of inputs from heat sensitive afferent remains to be determined.

These results indicate that laser stimulation not only reveals the responsiveness of dorsal horn neurons to noxious heat accurately, but this approach also provides additional information concerning the independent contribution of unmyelinated and myelinated nociceptors. To confirm the findings, we next asked whether dorsal horn neurons that did not respond to heat stimulation were also unresponsive to laser stimulation. In control animals, we found that none of the eight heat-insensitive neurons responded to the C fibre protocol laser stimulus, even at the highest laser intensity studied.

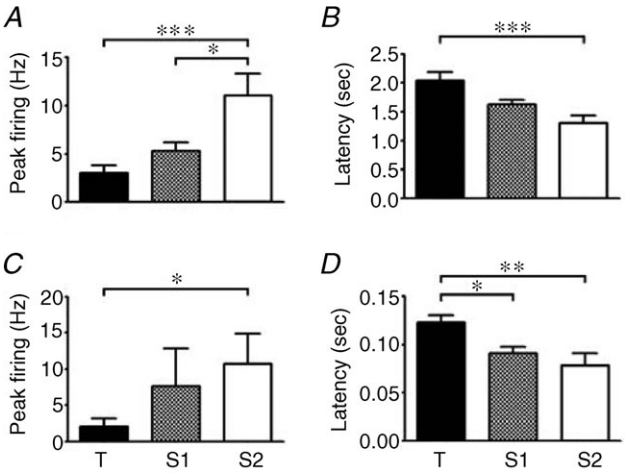


Figure 4. Responsiveness of superficial dorsal horn neurons to C fibre and Aδ fibre diode laser stimulation in vehicle-treated mice
A, coding (peak firing) using the C fibre stimulation protocol. First, the threshold intensity (T) to evoke firing was determined and then two incremental steps of suprathreshold intensity (S1 and S2) were studied (see Methods). There was a significant increase in firing as the intensity increased ($P < 0.001$). *B*, the latency in response to the C fibre protocol also decreased significantly at the higher laser intensities ($P < 0.01$). *C*, coding (peak firing) using the Aδ fibre stimulation protocol. As for the C fibre protocol, there was a significant increase in firing as laser intensity increased ($P < 0.05$). *D*, latency to firing decreased at higher stimulation intensities ($P < 0.01$). Data are presented as means + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, Bonferroni *post hoc* tests and one-way ANOVA.

However, we found that one neuron responded to the Aδ fibre protocol laser, with a threshold comparable to that of heat-responsive cells (in this case, 2000 mA).

Finally, we investigated the responses elicited by laser stimulation in capsaicin-treated mice. We found that the two superficial dorsal horn neurons that responded to contact heat stimulation following capsaicin treatment were also laser responsive but, as for contact heat, their firing properties in response to laser stimulation were unusual. Thus, one neuron had a very high C fibre activation threshold (1000 mA). The second heat-responsive cell responded only to the Aδ fibre protocol laser, but again the threshold was unusually high (4000 mA). Based on these findings, it appears that laser stimulation largely, if not completely, recapitulates findings using a contact heat stimulus.

In contrast, recordings from heat-unresponsive units in capsaicin-treated mice suggest that laser stimulation may provide a slightly more sensitive assay, especially in response to selective Aδ stimulation. Thus, although we found that none of 11 heat-insensitive neurons from the superficial dorsal horn of capsaicin-treated mice (6 NS, 3 WDR and 2 LT) and none of 12 from the region of lamina V (1 NS and 11 WDR) responded to the C fibre laser protocol, two in each group did respond to the Aδ protocol. The Aδ laser-responsive neurons in the superficial dorsal horn (both of which were WDR) did have unusually high thresholds (3100 and 3500 mA). However, the two heat-insensitive units in the deep dorsal horn (also WDR) had normal Aδ thresholds and coded for stimulus intensity. Overall, we conclude that ablation of TRPV1 afferents drastically reduces the firing of dorsal horn neurons in response to noxious heat; however, there appears to be a residual, albeit very limited, response to Aδ inputs, and this is detectable only using laser stimulation.

Ablation of MrgprD+ afferents selectively reduces the mechanical responsiveness of superficial dorsal horn neurons: differential effects on NS and WDR neurons

We previously reported that DTX-induced ablation of the MrgprD+ subset of dorsal root ganglion neurons reduced, but did not eliminate, the responsiveness of mice to noxious mechanical stimulation (Cavanaugh *et al.* 2009). Here we asked whether the behavioural phenotype in mice with an ablation of the MrgprD+ neurons is manifest at the level of nociresponsive neurons in the dorsal spinal cord. We recorded from 45 superficial dorsal horn neurons in vehicle-treated mice and 27 in DTX-treated mice. Although these mice have a mixed genetic background, response properties did not differ significantly from our findings in the C57Bl/6 mice used for the TRPV1+ afferent ablation studies. As illustrated in Fig. 5*A*, NS neurons predominated in the superficial dorsal horn in vehicle- (64.4%, 29 of 45) and DTX-treated mice (66.7%, 18 of 27). Wide dynamic range neurons constituted 31.1% (14 of 45) and 25.9% (7 of 27) of the total in vehicle- and DTX-treated mice, respectively. The remaining neurons were LTMs (2 in each group).

We found significant consequences of DTX-mediated ablation of the MrgprD+ subset of nociceptors on the mechanical responsiveness of superficial dorsal horn neurons. Although the mechanical properties of both NS and WDR neurons were affected, the nature of the effect differed between the two groups. Thus, for putative lamina I NS neurons, there was a significant decrease in both the peak firing rate and the total number of spikes in DTX-treated mice compared with vehicle-treated mice ($P < 0.01$ for peak firing rate and $P < 0.05$ for total number of spikes, by two-way ANOVA; Fig. 6*A*). On the contrary, although the mechanical response properties of polymodal WDR neurons in the superficial dorsal horn did not change ($P > 0.05$, two-way ANOVA; Fig. 6*B*), we observed a complete loss of the subset of WDR neurons that responds only to mechanical stimulation (WDR/M; 4 of 45 or 8.9% of all neurons recorded in vehicle-treated mice, and 0 of 27 or 0% in DTX-treated mice; $P < 0.05$, Z-test; Fig. 5*A*). Finally, consistent with the lack of effect of MrgprD+ neuron ablation on behavioural responses to noxious heat, we found no difference in the percentage of heat-responsive neurons in vehicle- (77.8%, 35 of 45)

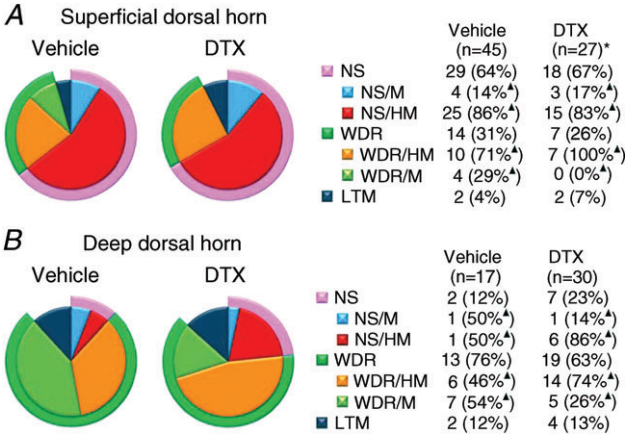


Figure 5. Categorization of mechanically responsive superficial and deep dorsal horn neurons in vehicle- and diphtheria toxin (DTX)-treated mice
A, these multilevel pie charts schematize the relative abundance of the different categories of presumptive lamina I neurons in vehicle- and DTX-treated mice, with their percentage relative to the total number of neurons recorded listed in the adjacent table. Note the loss of WDR/M units following DTX treatment (*$P < 0.05$, Z-test). *B*, multilevel pie charts schematize the relative abundance of the different categories of deep dorsal horn neurons, with their percentage relative to the total number of neurons recorded listed in the adjacent table. Note the reduction in WDR/M units, and the relative increase in heat-responsive units, following DTX treatment. Percentages marked with a filled triangle refer to the relative number of NS/M or NS/HM in the NS group, or of WDR/M or WDR/HM in the WDR group. Abbreviations are as for Fig. 2.

and DTX-treated mice (81.5%, 22 of 27; $P > 0.05$, Z-test Fig. 5*A*) and no difference in the coding of noxious heat stimulation by these neurons ($P > 0.05$, two-way ANOVA; Fig. 7*A* and *B*)

Ablation of MrgprD+ afferents has more limited effects on the mechanical responsiveness of deep dorsal horn neurons

Given that we previously demonstrated that one major output pathway of the non-peptidergic subset of nociceptors is via projection neurons in lamina V (Braz *et al.* 2005), we were especially interested in comparing the properties of nociresponsive neurons in the superficial and deep dorsal horn. To target putative lamina V neurons, we made recordings from 17 neurons in vehicle-treated mice and 30 in DTX-treated mice. A majority of the recorded neurons in both groups of animals were classified as WDR, but it appears that there was a small reduction in the overall percentage of WDR neurons in DTX-treated mice (63.3%; 19 of 30) compared with control mice (76.5%; 13 of 17; Fig. 5*B*). We also recorded an increase in the percentage of NS neurons (12% in vehicle-treated *vs.* in DTX-treated mice), but this change was not statistically significant,

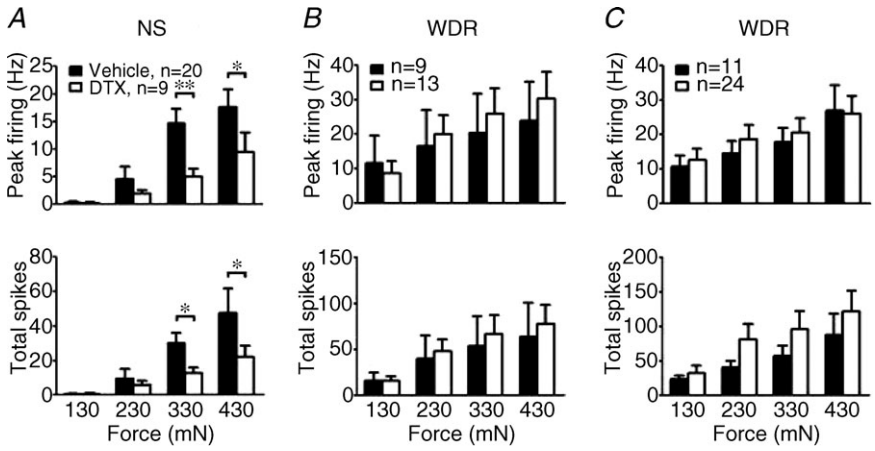


Figure 6. Ablation of MrgprD+ afferents reduces the mechanical responsiveness of superficial dorsal horn NS neurons
Peak firing (top panels) and total spikes (bottom panels) elicited by mechanical stimulation at the indicated intensities in superficial dorsal horn NS neurons (*A*), superficial dorsal horn WDR neurons (*B*) and deep dorsal horn WDR neurons (*C*) in vehicle-treated (filled columns) and DTX-treated mice (open columns). Two-way ANOVA showed significant reduction of both peak firing and total spikes in response to the graded mechanical stimuli in superficial dorsal horn NS neurons following DTX treatment compared with vehicle-treated animals ($*P < 0.05$ and $**P < 0.01$). Data are presented as means + SEM.

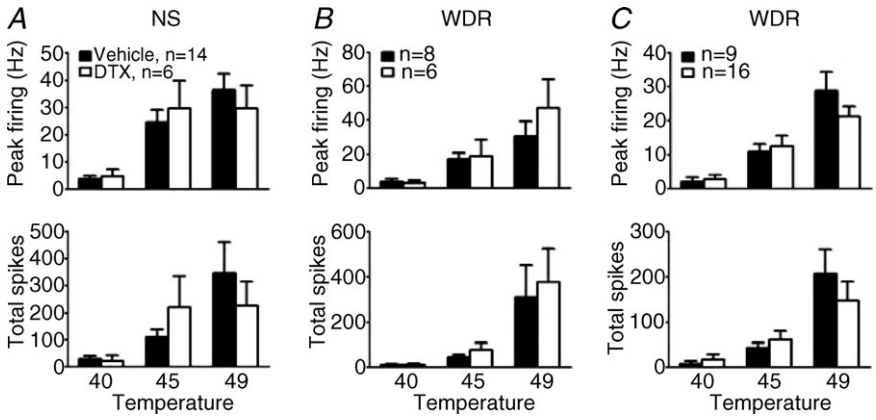


Figure 7. Ablation of MrgprD+ afferents does not alter heat responsiveness of spinal cord nociresponsive neurons
Peak firing (top panels) and total spikes (bottom panels) elicited by heat stimulation at the indicated intensities in superficial dorsal horn NS neurons (*A*), superficial dorsal horn WDR neurons (*B*) and deep dorsal horn WDR neurons (*C*) of vehicle-treated (filled columns) and DTX-treated mice (open columns). All groups encoded stimulus intensity with increased firing (one-way ANOVA), and there were no significant differences in the heat responsiveness between vehicle- and DTX-treated mice (two-way ANOVA). Data are presented as means + SEM.

undoubtedly because of the very low number of neurons that we identified in this group. Finally, the percentage of LTM neurons in the deep dorsal horn did not change (12% in vehicle-treated *vs.* 13% in DTX-treated mice).

We found that ablation of MrgprD+ afferents reduced the proportion of WDR neurons responsive only to mechanical stimulation (41.2% of all neurons recorded in vehicle-treated *vs.* 16.7% in DTX-treated mice; Fig. 5*B*). This reduction is similar to the observation in superficial dorsal horn, but in this case the difference was not statistically significant ($P = 0.07$, Z-test). Furthermore, this reduction occurred without any alteration of the firing properties (i.e. total firing and peak firing rate) of the WDR neurons in response to either mechanical or heat stimuli ($P > 0.05$, two-way ANOVA; Figs 6*C* and 7*C*). The low number of NS neurons in the deep dorsal horn of vehicle-treated mice did not allow for an analysis of the consequence of DTX treatment.

Neither capsaicin nor DTX treatment reduced the number of cold-responsive neurons in the superficial and deep laminae of the dorsal horn

As previous studies indicated that behavioural responses to cold stimulation were unchanged following ablation of either the TRPV1+ or MrgprD+ subset (Cavanaugh *et al.* 2009), we also assayed for both innocuous (20°C) and noxious (10°C) cold responsiveness of dorsal horn neurons in these different groups of mice. An example of a cold-responsive neuron is shown in Fig. 1*C*. We found that the relative percentage of cold-responsive neurons in capsaicin-treated mice in response to innocuous cold (4 of 25 tested in lamina I and 3 of 9 in lamina V) and noxious cold (6 of 16 in lamina I and 4 of 9 in lamina V) did not differ significantly from that in the vehicle-treated mice (for innocuous cold, 3 of 26 in lamina I and 4 of 10 in lamina V; and for noxious cold, 3 of 24 in lamina I and 4 of 10 in lamina V; $P > 0.05$, Fisher's exact test). Likewise, in DTX-treated mice, we found that the relative percentage of cold-responsive neurons (for innocuous cold, 2 of 7 tested in lamina I and 9 of 21 in lamina V; and noxious cold, 2 of 7 in lamina I and 7 of 21 in lamina V) did not differ from that in the vehicle-treated mice (innocuous cold, 5 of 18 in lamina I and 4 of 7 in lamina V; and noxious cold, 2 of 18 in lamina I and 4 of 7 in lamina V; $P > 0.05$, Fisher's exact test). As our search strategy was based on mechanical responsiveness and because we did not complete a detailed analysis of the coding properties of cold-responsive dorsal horn neurons, we cannot completely rule out a contribution of TRPV1+ or MrgprD+ afferents to the magnitude of cold responsiveness of dorsal horn neurons. However, because the number of neurons was comparable after the different ablation procedures, it is certain that a TRPV1- and MrgprD-negative subset of afferents must contribute to the cold responsiveness of dorsal horn neurons.

Discussion

We previously reported that ablation of the central terminals of the TRPV1+ subset of afferents eliminated the behavioural response to noxious heat, but had no effect on noxious mechanical or cold stimuli. By contrast, genetic ablation of the MrgprD+ afferents significantly increased von Frey mechanical thresholds, but did not change the response to noxious heat or cold (Cavanaugh *et al.* 2009). Here we report that ablation of the central terminals of the TRPV1+ subset largely eliminated the response of NS and WDR neurons in both the superficial and the deep dorsal horn to noxious heat stimulation, with no change in the response to noxious mechanical stimulation. Our new findings also expand on our previous observation that deletion of the *Trpv1* gene eliminated the heat responsiveness of WDR neurons in lamina V, but there was significant residual heat responsiveness of neurons in lamina I (Eckert *et al.* 2006). The more complete phenotype observed in the present study indicates that any residual noxious heat-evoked activity after *Trpv1* gene deletion must be mediated by an as yet unidentified heat transducer(s) that is also expressed in the TRPV1+ subset of nociceptors.

By contrast, ablation of the MrgprD+ subset reduced, but did not eliminate, the response of dorsal horn neurons to mechanical stimulation, without altering the response to noxious heat. Interestingly, the deficit in mechanical responsiveness was found only in heat-insensitive WDR neurons and in presumptive lamina I NS neurons. There was no change in the mechanical coding of polymodal WDR neurons in the superficial or deep dorsal horn.

We recognize that our search strategy would miss neurons that were exclusively activated by noxious heat. However, these have not yet been described in rodents, and even in the cat such neurons constitute less than 5% of projection neurons in lamina I (Craig *et al.* 2001). Furthermore, as there is no evidence that these neurons are driven by TRPV1-negative nociceptors, it is likely that this population would also be unresponsive after ablation of the TRPV1+ population of nociceptors. We conclude, therefore, that TRPV1+ afferents carry virtually all of the noxious heat-related signals to spinal cord neurons, both in the superficial and in the deep dorsal horn, and that these afferents are entirely dispensable for mechanical nociception (see also Bates *et al.* 2010). The fact that the number of cold-responsive dorsal horn neurons did not change also indicates that a population of afferents that does not express TRPV1 or MrgprD must transmit innocuous and noxious cold information to the dorsal horn.

As TRPA1-expressing afferents constitute a subset of the TRPV1 afferents (Story *et al.* 2003; Bautista *et al.* 2005; however, see Kwan *et al.* 2009), it follows that TRPA1, as we have previously argued (Bautista *et al.* 2006), is not required for acute innocuous or noxious cold sensibility. By contrast, the fact that the TRPM8-expressing population does not completely overlap with the TRPV1 population (McKemy *et al.* 2002; Peier *et al.* 2002; Takashima *et al.* 2007) suggests not only that the TRPV1 population is not required for normal cold sensibility, but also that the TRPM8 population is likely to be sufficient. Furthermore, we conclude that the MrgprD+ neurons contribute to at least two subsets of mechanoresponsive dorsal horn neurons; along with other, as yet unidentified afferents, they provide noxious mechanical input to superficial NS neurons, in addition to providing noxious mechanical input to superficial and deep dorsal horn mechanoresponsive WDR neurons.

Contribution of the non-peptidergic afferents to the processing of noxious mechanical inputs

As noted above in methods & electrophysiology, because we did not use antidromic activation to identify superficial dorsal horn neurons, we cannot definitively conclude that these recordings were obtained from neurons in lamina I. Based on our transneuronal tracing studies (Braz *et al.* 2005), we concluded that the isolectin B4 subset of afferents engages neurons in lamina I only minimally. It was, therefore, somewhat unexpected that we observed electrophysiological changes in superficial dorsal horn neurons following DTX treatment. One possible explanation is that loss of the WDR neurons responsive only to mechanical stimulation and the reduction of coding of NS neurons of the superficial dorsal horn reflect a loss of a polysynaptic input to lamina I neurons that is sufficiently limited to be undetectable in the tracing studies. Alternatively, the reduction in activity may reflect loss of descending facilitatory loops (Fields & Heinricher, 1985; Zhuo & Gebhart, 1997) that enhance the responsiveness of NS neurons to mechanical stimulation.

Several important insights follow from our observation of selective deficits in superficial dorsal horn NS neurons and in superficial and deep dorsal horn mechanosensitive WDR neurons following MrgprD+ neuron ablation. As both NS and WDR neurons are presumed to receive their high-threshold (noxious) mechanical input via polymodal C fibres and Aδ nociceptors (Price *et al.* 2003), the present results suggest either that a different complement of high-threshold afferents provides the nociceptive input to polymodal WDR and NS neurons or that there are redundant inputs to the WDR neurons that compensate when MrgprD+ afferents are ablated. Furthermore, the predominant effect of ablation of the MrgprD+ subset of nociceptors on NS neurons suggests that the behavioural deficit (increased von Frey threshold) produced in these animals is related to the firing of lamina I NS neurons. Unexpectedly, therefore, but consistent with a previous conclusion (Ogawa & Meng, 2009), it appears that lamina I neurons contribute both to ascending transmission of nociceptive messages and to acute noxious stimulus-evoked reflexes.

Laser *vs.* heat stimulation of noxious heat-responsive dorsal horn neurons

Given that the laser allowed us to adjust parameters to activate Aδ and C fibres selectively, we were able to delineate the distinct contribution of these two types of fibres to heat processing in the dorsal horn. We found that there is a significant convergence of C and Aδ inputs to nociresponsive neurons in both the superficial and the deep dorsal horn, but that the C fibre input predominates. Thus, all neurons responding to the Aδ protocol also responded to the C fibre protocol, but only 75% of the latter responded to the Aδ protocol. Based on this segregation, it will be of interest to use laser stimulation to assess the residual responsiveness of lamina I neurons after deletion of the *Trpv1* gene, so as to identify the subset of TRPV1+ afferents in which other noxious heat transducers must reside.

We found that all neurons that responded to noxious heat could be activated by laser stimulation, and these neurons coded both for heat and stimulus intensity. However, possibly due to the more intense and focused stimulation achievable with the laser, we observed a few instances in which neurons failed to respond to contact heat stimulation but did respond to a laser stimulus, using the Aδ protocol. Conceivably, the enhanced sensitivity using the laser also reflects the more rapid and uniform penetration though the skin provided by the 980 nm diode laser-generated heat (Le Bars *et al.* 2001), which allows for more efficient access to nociceptor terminals in deeper locations.

The loss of heat responsiveness after intrathecal capsaicin was, in almost every case, manifest as a loss of the response to laser stimulation. Thus, even with this more intense method of stimulation, we failed to detect a response to heat in the absence of TRPV1+ afferents. We did find a residual laser sensitivity of a few heat-insensitive dorsal horn neurons after capsaicin treatment but, as in vehicle-treated mice, this was true only when we used the Aδ stimulation protocol. As none of the MrgprD+ nociceptors is myelinated, these non-peptidergic afferents could not be the source of the residual laser sensitivity.

What is the significance of the polymodal nociceptor?

Given the polymodal properties of the vast majority of nociceptors, including both the TRPV1+ and MrgprD+ populations, the modality-specific deficits associated with selective ablation protocols are surprising, and call into question the prevailing view of how nociceptive information is processed in the periphery. If ablation of an apparently polymodal population results in modality-specific behavioural deficits (Cavanaugh *et al.* 2009) and a selective loss of heat or mechanical inputs to spinal cord neurons (present study), it is hard to understand how these afferents can be carrying polymodal information in any meaningful, physiologically relevant way.

The heat-specific deficits observed following ablation of TRPV1+ neurons are consistent with a recent report that a population of TRPV1+ afferents is exclusively heat activated (Lawson *et al.* 2008). What is particularly difficult to explain, however, is the apparent absence of a CNS contribution of the heat-responsive non-peptidergic subset of nociceptors (Jankowski *et al.* 2009; Rau *et al.* 2009). Although this population is not required for the behavioural response to noxious heat or for the induction of Fos in populations of dorsal horn neurons, we expected that the heat sensitivity of the MrgprD+ afferents would be manifest in a single-unit analysis of dorsal horn neurons. In fact, ablation of the MrgprD+ population had no effect on coding, peak firing or the total number of spikes of superficial or deep dorsal horn neurons in response to heat. Overall, we conclude that the 'pain transmission' system in the mouse consists of distinct populations of primary afferent nociceptors that provide a modality-specific contribution to the processing of nociceptive messages in the spinal cord dorsal horn and to the behavioural responses elicited by such messages.

This conclusion, in fact, is consistent with a recent report by Koerber and colleagues (2010) demonstrating that even though TRPV1-negative nociceptors can be sensitized by an inflammation-inducing stimulus, they are not sufficient to generate heat hyperalgesia in the absence of the TRPV1-expressing afferents. This scenario could arise if there is a significant difference in the firing rate in response to heating of the TRPV1+ and MrgprD+ nociceptors. For example, a heat-evoked low-frequency input from MrgprD+ afferents may not be sufficient to evoke action potentials in dorsal horn neurons after ablation of the TRPV1 afferents. This hypothesis could perhaps be tested by an intracellular analysis of excitatory postsynaptic potentials in dorsal horn nociresponsive neurons.

A final comment concerning the pattern of expression of TRPV1 is in order. Specifically, using an especially sensitive, genetically expressed TRPV1 reporter mouse as well as a very long-exposure radioactive *in situ* hybridization method, we found no TRPV1 expression in the CNS, with the exception of a population of cells in the region of the supramammilary nucleus of the rostral midbrain (Cavanaugh *et al.* 2011). Despite our findings, there continue to be reports of functional TRPV1 in the brain and, most recently, in the spinal cord dorsal horn (Kim *et al.* 2012). In our opinion, verification of the CNS claims awaits identification and cloning of the CNS TRPV1 transcripts. It is important to emphasize, however, that our conclusions concerning the behaviourally relevant specificity of the processing of heat and mechanical pain information would not differ were there dorsal horn neurons that express TRPV1. Intrathecal capsaicin would undoubtedly kill these TRPV1-expressing dorsal horn neurons, as well as the TRPV1-expressing central terminals, which would suggest that the basis of the modality specificity mediated by neurons that express TRPV1 extends into the circuits within the dorsal horn.

References

Basbaum AI, Bautista DM, Scherrer G & Julius D (2009). Cellular and molecular mechanisms of pain. *Cell* **139**, 267–284.

Bates BD, Mitchell K, Keller JM, Chan CC, Swaim WD, Yaskovich R, Mannes AJ & Iadarola MJ (2010). Prolonged analgesic response of cornea to topical resiniferatoxin, a potent TRPV1 agonist. *Pain* **149**, 522–528.

Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI & Julius D (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* **124**, 1269–1282.

Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O, Hogestatt ED, Julius D, Jordt SE & Zygmunt PM (2005). Pungent products from garlic activate the sensory ion channel TRPA1. *Proc Natl Acad Sci U S A* **102**, 12248–12252.

Braz JM & Basbaum AI (2010). Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. *Pain* **150**, 290–301.

Braz JM, Nassar MA, Wood JN & Basbaum AI (2005). Parallel "pain" pathways arise from subpopulation of primary afferent nociceptor. *Neuron* **47**, 787–793.

Cavanaugh DJ, Chesler AT, Braz JM, Shah NM, Julius D & Basbaum AI (2011). Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. *J Neurosci* **31**, 10119–10127.

Cavanaugh DJ, Lee HS, Lo LC, Shields SD, Zylka MJ, Basbaum AI & Anderson DJ (2009). Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc Natl Acad Sci U S A* **106**, 9075–9080.

Craig AD, Krout K & Andrew D (2001). Quantitative response characteristics of thermoreceptive and nociceptive lamina I spinothalamic neurons in the cat. *J Neurophysiol* **86**, 1459–1480.

Cuellar JM, Manering NA, Klukinov M, Nemenov MI & Yeomans DC (2010). Thermal nociceptive properties of trigeminal afferent neurons in rats. *Mol Pain* **6**, 39.

Eckert WA, Julius D & Basbaum AI (2006). Differential contribution of TRPV1 to thermal responses and tissue injury-induced sensitization of dorsal horn neurons in laminae I and V in the mouse. *Pain* **126**, 184–197.

Fields HL & Heinricher MM (1985). Anatomy and physiology of a nociceptive modulatory system. *Philos Trans R Soc Lond B Biol Sci* **308**, 361–374.

In-Silico.net (2011). *In-Silico Online, Medical- and Bio-Statistics*. In-Silico Online (http://in-silico.net/statistics).

Jankowski MP, Lawson JJ, McIlwrath SL, Rau KK, Anderson CE, Albers KM & Koerber HR (2009). Sensitization of cutaneous nociceptors after nerve transection and regeneration: possible role of target-derived neurotrophic factor signaling. *J Neurosci* **29**, 1636–1647.

Kim, YH, Back, SK, Davies AJ, Jeong H, Jo HG, Chung G, Na HS, Bae YC, Kim SJ, Kim JS & Oh SB (2012). TRPV1 in GABAergic interneurons mediates neuropathic mechanical allodynia and disinhibition of the nociceptive circuitry in the spinal cord. *Neuron* **74**, 640–647.

Koerber HR, McIlwrath SL, Lawson JJ, Malin SA, Anderson CE, Jankowski MP & Davis BM (2010). Cutaneous C-polymodal fibers lacking TRPV1 are sensitized to heat following inflammation, but fail to drive heat hyperalgesia in the absence of TPV1 containing C-heat fibers. *Mol Pain* **6**, 58.

Kwan KY, Glazer JM, Corey DP, Rice FL & Stucky CL (2009). TRPA1 modulates mechanotransduction in cutaneous sensory neurons. *J Neurosci* **29**, 4808–4819.

Lawson JJ, McIlwrath SL, Woodbury CJ, Davis BM & Koerber HR (2008). TRPV1 unlike TRPV2 is restricted to a subset of mechanically insensitive cutaneous nociceptors responding to heat. *J Pain* **9**, 298–308.

Le Bars D, Gozariu M & Cadden SW (2001). Animal models of nociception. *Pharmacol Rev* **53**, 597–652.

Light AR (1992). *The Initial Processing of Pain and its Descending Control: Spinal and Trigeminal System*, ed. Gildenberg PL, Vol 12, 306. Karger, Basel.

McKemy DD, Neuhausser WM & Julius D (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52–58.

Mazario J & Basbaum AI (2007). Contribution of substance P and neurokinin A to the differential injury-induced thermal and mechanical responsiveness of lamina I and V neurons. *J Neurosci* **27**, 762–770.

Mishra SK, Tisel SM, Orestes P, Bhangoo SK & Hoon MA (2011). TRPV1-lineage neurons are required for thermal sensation. *EMBO J* **30**, 582–593.

Mitchell K, Bates BD, Keller JM, Lopez M, Scholl L, Navarro J, Madian N, Haspel G, Nemenov MI & Iadarola MJ (2010). Ablation of rat TRPV1-expressing Adelta/C-fibers with resiniferatoxin: analysis of withdrawal behaviors, recovery of function and molecular correlates. *Mol Pain* **6**, 94.

Ogawa A & Meng ID (2009). Differential effects of the cannabinoid receptor agonist, WIN 55,212–2, on lamina I and lamina V spinal trigeminal nucleus caudalis neurons. *Pain* **141**, 269–275.

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S & Patapoutian A (2002). A TRP channel that senses cold stimuli and menthol. *Cell* **108**, 705–715.

Price DD, Greenspan JD & Dubner R (2003). Neurons involved in the exteroceptive function of pain. *Pain* **106**, 215–219.

Rau KK, McIlwrath SL, Wang H, Lawson JJ, Jankowski MP, Zylka MJ, Anderson DJ & Koerber HR (2009). MrgprD enhances excitability in specific populations of cutaneous murine polymodal nociceptors. *J Neurosci* **29**, 8612–8619.

Shields SD, Cavanaugh DJ, Lee H, Anderson DJ & Basbaum AI (2010). Pain behavior in the formalin test persists after ablation of the great majority of C-fiber nociceptors. *Pain* **151**, 422–429.

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S & Patapoutian A (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112**, 819–829.

Stucky CL & Lewin GR (1999). Isolectin B_4-positive and -negative nociceptors are functionally distinct. *J Neurosci* **19**, 6497–6505.

Takashima Y, Daniels RL, Knowlton W, Teng J, Liman ER & McKemy DD (2007). Diversity in the neural circuitry of cold sensing revealed by genetic axonal labeling of transient receptor potential melastatin 8 neurons. *J Neurosci* **27**, 14147–14157.

Tzabazis A, Klyukinov M, Manering N, Nemenov MI, Shafer SL & Yeomans DC (2005). Differential activation of trigeminal C or Aδ nociceptors by infrared diode laser in rats: behavioral evidence. *Brain Res* **1037**, 148–156.

Urch CE, Donovan-Rodriguez T & Dickenson AH (2003). Alterations in dorsal horn neurones in a rat model of cancer-induced bone pain. *Pain* **106**, 347–356.

Yeomans DC & Proudfit HK (1996). Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: electrophysiological evidence. *Pain* **68**, 141–150.

Zhuo M & Gebhart GF (1997). Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. *J Neurophysiol* **78**, 746–758.

Zylka MJ, Dong X, Southwell AL & Anderson DJ (2003). Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. *Proc Natl Acad Sci U S A* **100**, 10043–10048.

Zylka MJ, Rice FL & Anderson DJ (2005). Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to MrgprD. *Neuron* **45**, 17–25.

Author contributions

J.Z., D.J.C., M.I.N. and A.I.B. designed research; J.Z., D.J.C. and M.I.N performed research; J.Z, D.J.C., M.I.N. and A.I.B. analysed data; J.Z., D.J.C. and A.I.B. wrote the paper; and all authors approved the final version.

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